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TITLE: Prevention and Treatment of Neurofibromatosis Type 1-Associated Malignant Peripheral Nerve Sheath Tumors

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14. ABSTRACT
The most common cause of death in Neurofibromatosis Type 1 (NF1) patients is malignant peripheral nerve sheath tumor (MPNST). MPNSTs are aggressive Schwann cell-derived neoplasms that typically arise from precursor lesions such as plexiform neurofibromas. Although gross total resection of MPNSTs is potentially curative, this occurs in only a small minority of cases. Radiotherapy and chemotherapy may inhibit local recurrence but have almost no effect on patient mortality. NF1 patients have an approximate 10% lifetime risk of developing an MPNST and this risk increases to approximately 30% in patients with plexiform neurofibromas. Thus, development of safe and effective MPNST preventative therapies could have an important impact on NF1 patient morbidity and mortality. In this grant, we are testing the hypothesis that chronic administration of agents that promote apoptosis and/or inhibit pro-survival autophagy will inhibit MPNST formation and progression in transgenic mouse models of MPNST. Specifically, we are examining the mechanisms of action and *in vivo* utility of two classes of drugs, BH3 mimetics and lysosomotropic agents, on MPNSTs. The drugs that we are testing are approved for human use and could be rapidly advanced into human MPNST clinical trials if our pre-clinical testing yields positive results.

15. SUBJECT TERMS
Apoptosis; autophagy; lysosomotropic agents; Bcl2 family members

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Title of Grant: Prevention and Treatment of Neurofibromatosis Type 1- Associated Malignant Peripheral Nerve Sheath Tumors

Award #: W81XWH-14-1-0073
Principal Investigator: Kevin A. Roth, MD, PhD
Annual Report: 04/01/2014 – 03/31/2015

1. Introduction

Neurofibromatosis type 1 (NF1) has a frequency of approximately one in 3,000 humans and decreases life expectancy by ten to twenty years. Malignant peripheral nerve sheath tumors (MPNSTs) are the leading cause of death in NF1 patients and typically arise from NF1-associated precursor lesions. NF1 patients have an approximate 10% lifetime risk of developing a MPNST and this risk may be as high as 30% in NF1 patients with symptomatic plexiform neurofibromas. MPNSTs afflict NF1 patients in the prime of their lives, median age at diagnosis being approximately 40 years, and have a poor prognosis with median disease specific survival of approximately five to eight years. Gross total surgical resection is the only curative therapy and is unobtainable in the vast majority of patients. Radiotherapy and chemotherapy have proven largely ineffective in extending MPNST patient survival. Tumor formation and malignant progression are both dependent on the ability of tumor cells to evade normal cell death inducing stimuli. Numerous studies have shown that overexpression of anti-apoptotic Bcl-2 family members such as Bcl-2, Bcl-XL and MCL-1 can decrease tumor cell sensitivity to both radiotherapy and chemotherapy. Small molecule inhibitors of anti-apoptotic Bcl-2 proteins which have a functional BH3 domain, so called "BH3 mimetics", can potentiate tumor cell sensitivity to standard chemotherapeutic agents. Similarly, cytoprotective autophagy is commonly increased in tumor cells permitting these cells to survive in nutrient poor and hypoxic conditions that would kill normal cells. Cytoprotective autophagy can be inhibited by lysosomotropic agents such as chloroquine (CQ) which inhibit lysosome degradation of autophagic vacuoles and their contents. To date, no studies of combined BH3 mimetic and lysosomotropic agents have examined their potential utility as MPNST chemopreventive agents in either animal models or in NF1 patients.

2. Keywords

Apoptosis
Autophagy
Lysosomotropic agents
Bcl2 family members

3. Accomplishments

What were the major goals of the project?

1. To determine therapeutic effects of BH3 mimetics and lysosomotropic agents to inhibit Schwann cell hyperproliferation, MPNST formation and progression in transgenic mouse models.
2. To determine the effects and mechanisms of action of BH3 mimetics and lysosomotropic agents, alone and in combination, on NF1 patient-derived MPNST cell lines *in vitro*.

What was accomplished under these goals?

In the first year of this project, we met all of our proposed initial objectives including obtaining appropriate regulatory approval, expanding our colony of transgenic mice, initiating *in vivo* studies on P_0-GGFβ3 young adult mice and P_0-GGFβ3x $p53^{+/-}$ mice, and expanding cultures of human-derived MPNST cell lines for *in vitro* studies of BH3 mimetics and lysosomotropic agents. Our initial results are presented below.

Effects of BH3 mimetics and lysosomotropic agents on Schwann cell proliferation in the sciatic nerves of young adult P_0-GGFβ3 transgenic mice. We began these studies by breeding cohorts of P_0-GGFβ3 mice onto a pure C57BL/6J genetic background and verifying the presence of Schwann cell hyperproliferation in the sciatic nerve at five to six weeks of age as evidenced by increased Ki-67 and BrdU labeling. (Figure One) Having confirmed our previously published results on P_0-GGFβ3 Schwann cell hyperproliferation, we are now proceeding to assess the *in vivo* effects of daily delivery of two lysosomotropic agents, chloroquine (CQ) and quinacrine (AQ), each at 2mg/kg/day, and two BH3 mimetics, AT-101 and ABT-737, each at 5mg/kg/day. The initial experiment has been completed and we are now staining and performing cell counts on the collected tissues.

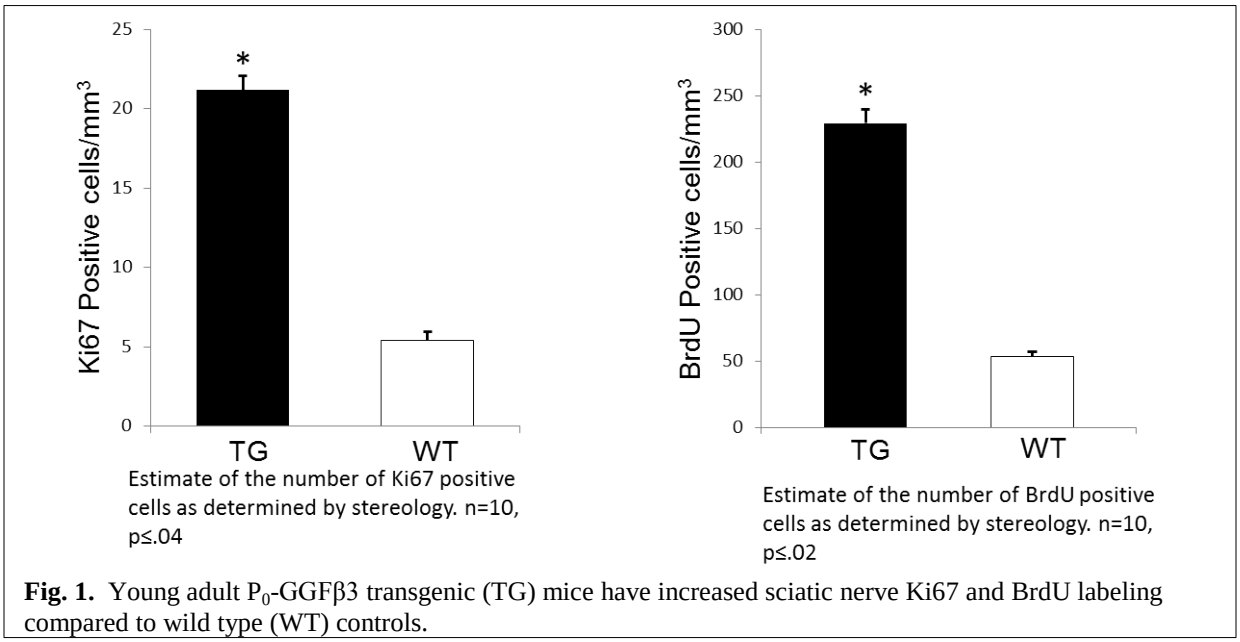

Fig. 1. Young adult P_0-GGFβ3 transgenic (TG) mice have increased sciatic nerve Ki67 and BrdU labeling compared to wild type (WT) controls.

Determine if BH3 mimetics and lysosomotropic agents inhibit the occurrence of neurofibromas in P_0-GGFβ3 transgenic mice on a mixed C57BL/6J x SJL/J genetic background. We began these studies by generating transgenic mice on the required mixed genetic background since we had been maintaining the P_0-GGFβ3 transgenic mice on a pure C57BL/6J background. Based on our previously reported results, we know that genetic background has a significant effect on the frequency of spontaneous neurofibromas and MPNSTs in this model. Thus, we need to verify that the genetically mixed transgenic mice that we generate have a frequency and distribution of tumors similar to the originally generated transgenic mice. Breeding and analysis of mixed C57BL/6J x SJL/J P_0-GGFβ3 mice is underway and must be completed prior to drug studies. We anticipate that sufficient numbers of mice will be available in year two to begin our studies of chronic lysosomotropic agent and BH3 mimetic treatment.

Determine if BH3 mimetics and lysosomotropic agents inhibit the formation of low grade and high grade MPNSTs in P_0-GGFβ3/Trp53$^{+/-}$ C57BL/6J transgenic mice. We generated sufficient numbers of transgenic mice (>20 per group) to begin testing the effects of chronic CQ and AT-101 (alone and in combination) on tumor formation and mortality in P_0-GGFβ3/Trp53$^{+/-}$ mice. We have confirmed our previous findings that P_0-GGFβ3/Trp53$^{+/-}$ mice develop aggressive MPNSTs starting around 150 days of life and we have been performing thorough necropsies on each animal. Since tumor formation can occur anywhere from 150-300 days, this study is still underway and will require another three to six months before we have completed survival data and histopathological analyses on tumor frequency and aggressiveness. Based on those results, we will modify the dosage and delivery schedule of CQ and AT-101 and/or extend the study to additional BH3 mimetics and lysosomotropic agents as discussed in our original application.

Define the molecular mechanism by which BH3 mimetics and lysosomotropic agents lead to MPNST cell death using NF1 patient-derived MPNST cell lines. We have focused much of our effort in year one of the

grant on the proposed *in vitro* studies of MPNST cell lines while we generate sufficient numbers of transgenic mice for our *in vivo* experiments described above.

Our initial objective was to extend our analysis of the effects of AT-101 on BNIP3 and autophagy to other BH3 mimetics. As described in our original application, we found that the BH3 mimetic AT-101 stimulated autophagy and BNIP3 expression and that these effects were important for causing AT-101-induced MPNST cell death. Thus, we analyzed the effects of additional BH3 mimetics, including ABT-737, obatoclax and sabutoclax, on autophagic vacuole accumulation and BNIP3 expression levels. We found that AT-101 uniquely increased BNIP3 protein levels on western blots of BH3 mimetic exposed MPNST cell lines (Figure Two). We determined that this effect of AT-101 was due to its ability to chelate iron and not its BH3 mimetic property. Given the lack of effect of the other BH3 mimetics on BNIP3 expression, we can conclude that the MPNST cytotoxic effects of BH3 mimetics do not require enhanced BNIP3 expression.

We next compared the effects of AT-101, ABT-737, ABT-263, obatoclax and sabutoclax on the accumulation of autophagic vacuoles in treated MPNST cell lines. We found that AT-101 and obatoclax, but not ABT-737, ABT-263 and sabutoclax, increased the levels of LC3II, a well accepted marker of autophagic vacuoles, in western blots of MPNST cell line extracts (Figure Two). These results indicate that altered autophagy may play some role in AT-101 and obatoclax-induced MPNST cell death but that altered autophagy is not a property of all BH3 mimetics.

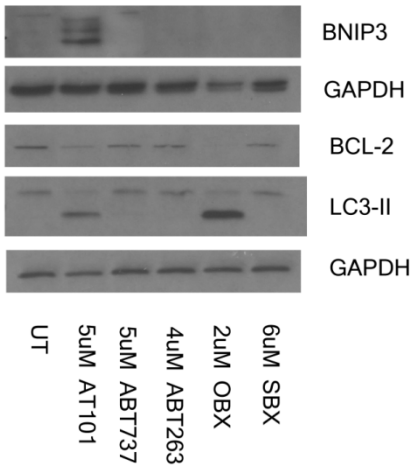

Fig. 2. Western blot analysis of lysates from T265 cells 24 hours after treatment with BH3 mimetics shows an increase in BNIP3 with AT-101 and an increase in LC3-II with AT-101 and Obatoclax.

We began experiments to determine if combined administration of BH3 mimetics and lysosomotropic agents would enhance MPNST cytotoxicity using a panel of NF1 patient-derived cell lines. We initially focused our studies on T265 MPNST cells and observed significantly greater cell killing when we combined BH3 mimetics (AT-101 and ABT-737) with lysosomotropic agents (CQ and QA) (Figure Three). However, when we extended the analysis to two additional MPNST cell lines, S462 and 8814, the enhanced cytotoxicity of combined treatment was not significant (data not shown) even though both cell lines were sensitive to BH3 mimetics and lysosomotropic agents individually. These observations point out the importance of testing these agents in multiple MPNST cell lines and the significance of tumor specific responsiveness to chemotherapeutic agents. In year two of the grant, we will perform additional experiments to attempt to understand the molecular mechanisms that determine whether or not an MPNST cell line will respond to combinational therapy.

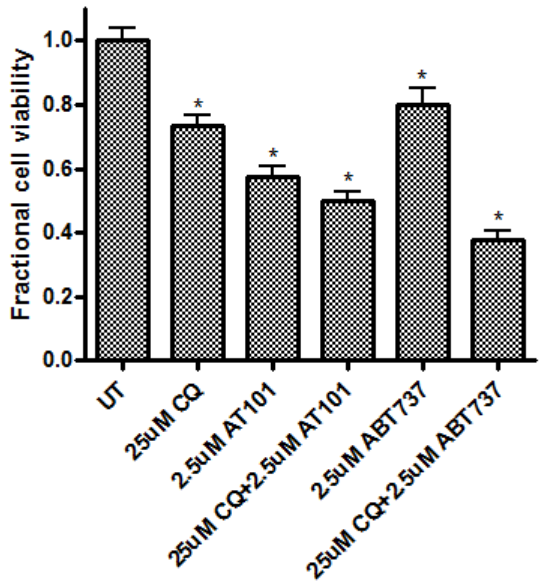

Fig. 3. Chloroquine combined with AT-101 or ABT737 causes a decrease in cell viability at 48 hours in T265 cells. (*p<0.001 versus untreated cells)

To gain additional insights into the mechanisms of BH3 mimetic-induced MPNST cytotoxicity, we examined the effects of AT-101 and other BH3 mimetics on CXCL12 gene expression in multiple MPNST cell lines. It was recently reported that CXCL12 acting on its receptor CXCR4 participated in an autocrine growth loop that enhanced proliferation and aggressiveness of NF1 patient-derived MPNST cells *in vitro* and *in vivo*. We found that AT-101 and other BH3 mimetics caused a marked reduction in MPNST expression of CXCL12 mRNA and protein (Figure Four). These preliminary studies point to another possible mechanisms by which BH3 mimetics might affect MPNST formation and progression, i.e. inhibition of the CXCL12/CXCR4 autocrine signaling pathway. Additional studies are needed to determine the significance of these preliminary observations.

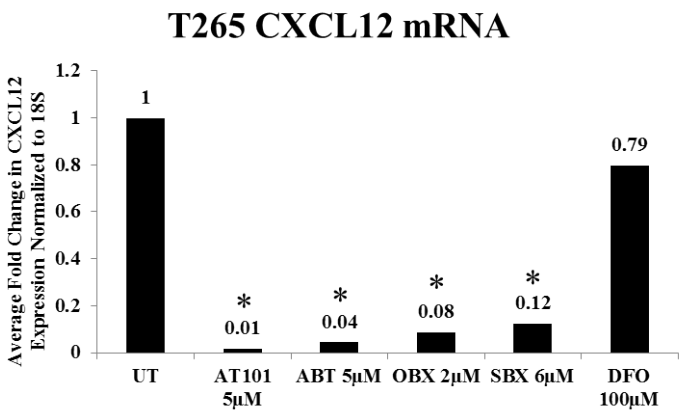

Fig. 4. AT-101 and other BH3 mimetics dramatically suppress CXCL12 mRNA expression in MPNST cells. The iron chelator DFO does not have this effect.

Key Research Accomplishments

- Established the transgenic mouse model systems required for *in vivo* testing of the effects of BH3 mimetics and lysosomotropic agents on MPNST formation and progression.
- Tested the ability of BH3 mimetics and lysosomotropic agents, alone and in combination, to kill NF1-assocaited MPNSTs *in vitro*.
- Determined that BNIP3 and autophagic vacuole accumulation are not required for the MPNST cytotoxic action of BH3 mimetics.
- Discovered that BH3 mimetics dramatically down regulate expression of CXCL12 mRNA which may disrupt the previously described NF1-associated MPNST CXCL12/CXCR4 autocrine growth axis.

What opportunities for training and professional development has the project provided?
Dr. Roth and Dr. Carroll met with colleagues at the recent Experimental Biology 2015 Meeting in Boston and discussed this work.

How were the results disseminated to communities of interest?
Dr. Roth presented lectures on this work at several institutions and at national meetings.

What do you plan to do during the next reporting period to accomplish the goals?
We will proceed with the previously proposed *in vivo* studies and expand our *in vitro* studies to include further evaluation of the CXCL12/CXCR4 pathway in MPNST progression.

4. Impact

What was the impact on the development of the principal discipline(s) of the project?
BH3 mimetics and lysosomotropic agents are potentially useful new compounds for inhibiting MPNST formation and progression in NF1 patients but they require additional testing in animal models and further definition of their molecular mechanisms of action on MPNST cells. Our first year of funding has laid a solid foundation for these additional studies and we will proceed with the previously proposed *in vitro* and *in vivo* experiments as well as further investigating our novel observation that BH3 mimetics suppress CXCL12 expression in MPNST cell lines.

What was the impact on other disciplines?
Nothing to Report

What was the impact on technology transfer?
Nothing to Report

5. Changes/Problems
Nothing to report

6. Products

Publications, conference papers, and presentations

Journal publications
We are preparing manuscripts describing our findings but require additional time to replicate and extend key observations prior to submission.

Books or other non-periodical, one-time publications
Nothing to Report

Other publications, conference papers, and presentations
Presentations:
04-29-2014 – University of California, San Diego – "Autophagy Targeted Therapy for Malignant Glial Neoplasms"
09-17-2014 – Italian Pathology Society (SIPMET) Annual Meeting, Palermo, Italy – Plenary Lecture – "Autopsy Targeted Therapy for Malignant Glial Neoplasms"
03-29-2015 – ASIP 2015 Annual Meeting at Experimental Biology – Boston – "AT-101 Don-regulates CXCL12 mRNA and Secreted Protein in Malignant Peripheral Nerve Sheath Tumor Cells"

Website(s) or other Internet site(s)
Nothing to Report

Technologies or techniques
Nothing to Report

Inventions, patent applications, and/or licenses
Nothing to Report

Other Products
Nothing to Report

7. Participants & Other Collaborating Organizations

What individuals have worked on the project?
Kevin A. Roth, MD, PhD – No Change
Steven L. Carroll, MD, PhD – No Change
Barbara J. Klocke, MD – No Change

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to report.

What other organizations were involved as partners?
Nothing to Report

8. Special Reporting Requirements
None

9. Appendices
None